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INFECTION AND IMMUNITY, Oct. 1993, p. 4217–4224
0019-9567/93/104217-08$02.00/0

Vol. 61, No. 10

Use of Transformation To Construct Antigenic Hybrids of the Class 1 Outer Membrane Protein in *Neisseria meningitidis*

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Received 17 May 1993/Returned for modification 25 June 1993/Accepted 22 July 1993

The class 1 protein of *Neisseria meningitidis* is an important component of candidate outer membrane vaccines against meningococcal meningitis. This porin protein contains two variable regions which determine subtype specificity and provide binding sites for bactericidal monoclonal antibodies. To determine the contribution of each of these variable regions in the induction of bactericidal antibodies, a set of isogenic strains differing only in their class 1 epitopes was constructed. This was done by transformation of meningococcal strain H44/76 with cloned class 1 genes and selection of the desired epitope combinations in a colony blot with subtype-specific monoclonal antibodies. When used for the immunization of mice, outer membrane complexes induced bactericidal antibodies only against meningococcal strains sharing at least one of their class 1 epitopes. The results demonstrate that the P1.2 and P1.16 epitopes, normally located in the fourth exposed loop of the protein, efficiently induce bactericidal antibodies independently of the particular sequence in the first variable region. The P1.5 and P1.7 epitopes, normally located in the first exposed loop, were found to induce lower bactericidal titers. Hybrid class 1 outer membrane proteins were constructed by inserting oligonucleotides encoding the P1.7 and P1.16 epitopes into the *porA* gene. In this way, we obtained a set of strains which carry the P1.5 epitope in loop 1, P1.2 in loop 4, and P1.7 and P1.16 (separately or in combination) in either loop 5 or loop 6. The additional epitopes were found to be exposed at the cell surface. Outer membrane complexes from several of these strains were found to induce a bactericidal response in mice against the inserted epitopes. These results demonstrate that it is feasible to construct meningococcal strains carrying multivalent class 1 proteins in which multiple subtype-specific epitopes are present in different cell surface-exposed loops.

Neisseria meningitidis is a human pathogen of worldwide significance. Vaccines containing the capsular polysaccharide provide limited protection against infection caused by strains of serogroups A and C. Immunity is, however, of short duration, and infants fail to respond. Polysaccharide vaccines do not induce protection against group B organisms, the predominant cause of infection in many countries, because of the poor immunogenicity of the group B polysaccharide in humans. Therefore, vaccines based on outer membrane proteins are currently being evaluated for their efficacy in preventing meningococcal disease (11, 36). Recent field trials in humans with such vaccines have demonstrated at least partial protection against group B infection (4, 7).

The porins are among the most abundant proteins present in the meningococcal outer membrane, and unlike several other major surface antigens, they do not undergo antigenic drift during infection. They function by creating pores through which small hydrophilic solutes can pass in a diffusion-like process. All meningococcal strains contain either a class 2 or class 3 protein, both of which form pores with anion selectivity (5). The class 1 protein is expressed by almost all meningococcal isolates, although there is variation in the level of expression (24). It forms pores which display cationic selectivity (30). Antigenic diversity of class 2/3 and class 1 proteins forms the basis for division of meningococcal strains into different serotypes and subtypes, respectively (12).

Monoclonal antibodies (MAbs) directed against subtype-specific epitopes on class 1 protein have been shown to be particularly effective in bactericidal assays and in conferring protection in an animal model (27, 28). Also, after immunization of mice with outer membrane complexes (OMCs), bactericidal activity is mainly directed against this protein (32). Thus, OMCs containing class 1 protein are serious candidates for a meningococcal vaccine. A major problem with this approach, i.e., the limited range of protection, might be overcome by constructing a strain expressing multiple class 1 proteins of different subtypes and lacking other, nonprotective outer membrane proteins (32).

Sequence comparison of different class 1 subtypes has shown that the major variation is confined to two discrete regions (17, 18). The epitopes recognized by bactericidal MAbs were localized to either one of these variable domains (18, 31). In a model for the topology of neisserial porins, these sequences are located in the first and fourth surface-exposed loops (31). In the present study, we have investigated the extent to which these two regions act independently in the generation of bactericidal antibodies. For this purpose, a set of isogenic strains differing only in their combination of class 1 epitopes was constructed through transformation. In the same way, strains containing insertions in other, normally more conserved loops were also made. These newly inserted epitopes were found to be exposed at the cell surface, demonstrating that this is a feasible way to construct strains with multivalent class 1 proteins.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists the meningococcal strains used in this study. They were grown

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TABLE 1. Meningococcal strains

Strain	Relevant characteristics	Reference
H44/76	B:15:P1.7,16	13
2996	B:2b:P1.5,2	25
MC50	C:nt:P1.16	3
HIII5	Class 3-deficient mutant of H44/76	30
1-2	HIII5 with second *porA* gene	32
TR52	H44/76 transformed with pCO14 to P1.5,2	This study
TR72	H44/76 transformed with pCO3 to P1.7,2	This study
TR516	TR52 transformed with pCO6 to P1.5,16	This study
TR7216	TR72 with P1.16 epitope inserted in loop 5	This study
K007	TR52 with P1.7 epitope inserted in loop 5	This study
K016	TR52 with P1.16 epitope inserted in loop 5	This study
K716	TR52 with P1.7 and P1.16 epitopes inserted in loop 5	This study
J007	TR52 with P1.7 epitope inserted in loop 6	This study
J016	TR52 with P1.16 epitope inserted in loop 6	This study
J716	TR52 with P1.7 and P1.16 epitopes inserted in loop 6	This study

overnight at 37°C on GC medium base (Difco) plates supplemented with IsoVitaleX in a humid atmosphere containing 5% CO_2. The medium used for growth in liquid culture contained, per liter, 1.3 g of glutamic acid, 0.02 g of cysteine, 2.5 g of $Na_2HPO_4 \cdot 2H_2O$, 0.09 g of KCl, 6.0 g of NaCl, 1.25 g of NH_4Cl, 0.6 g of $MgSO_4 \cdot 7H_2O$, 5.0 g of glucose, and 2.0 g of yeast extract (pH 7.8) and was filter sterilized. *Escherichia coli* NM522, used for the propagation of plasmids, was grown in LB medium (26) containing ampicillin (100 μg/ml) and, when necessary, 1 mM isopropylthiogalactopyranoside (IPTG).

Recombinant DNA techniques. Plasmids were constructed by using standard recombinant DNA techniques (26). Sticky ends were made blunt by incubation with T4 DNA polymerase, as prescribed by the manufacturer (Boehringer Mannheim). Restriction fragments were purified from low-melting-point agarose gels (NuSieve GTG agarose; FMC BioProducts). The polymerase chain reaction (PCR) was performed for 30 cycles of 1 min at 95°C, 1.5 min at 42°C, 1 min at 55°C, and 1 min at 72°C on a Bio-Med Thermocycle 60. The PCR buffer (10×) contained 100 mM Tris · HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, and 0.1% gelatin. *Taq* polymerase was purchased from Perkin-Elmer Cetus.

Construction of plasmids. The plasmid vector used for all constructs was pTZ19R (21). Cloning and sequencing of the *porA* gene from strains 2996, H44/76, and MC50 have been described previously (3, 18, 31). A diagram of the *porA* plasmids used is shown in Fig. 1. Plasmids pCO2 and pCO3 both contain a 1.9-kb *Eco*RI fragment with the C-terminal part of the *porA* gene of strain 2996 (subtype P1.5,2) in opposite orientations. A 29-bp deletion in the polylinker of pCO3 was made by digesting with *Sal*I and *Eco*RI, flushing the sticky ends with T4 DNA polymerase, and religating. This resulted in plasmid pCO20, which has an in-frame fusion between the *lacZ'* gene and the *porA* gene starting from residue 100 and a now unique *Kpn*I site located in the part of the gene encoding loop 5. The complete strain 2996 *porA* gene is present in plasmid pCO14 on a 2.8-kb *Xba*I-*Eco*RI fragment. In pCO6, the C-terminal part of *porA* from strain MC50 (subtype P1.16) is contained on a 1.3-kb *Eco*RI-*Xba*I fragment.

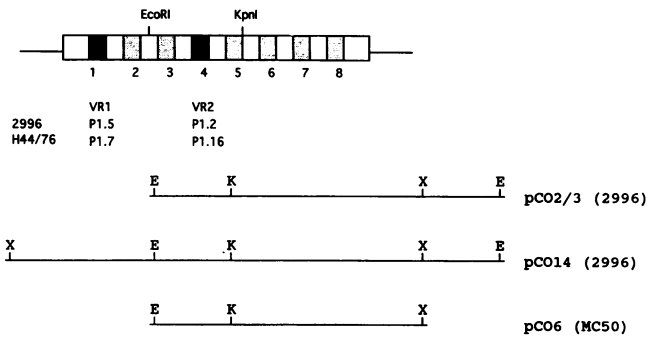

FIG. 1. Schematic representation of the *porA* gene. Shaded areas indicate the eight surface-exposed regions. Restriction fragments carried by the various plasmids used in this study are indicated below, showing what portion of the *porA* gene is contained in each. Plasmids pCO2 and pCO3 contain the same fragment but in opposite orientations. Restriction sites indicated are for *Eco*RI (E), *Kpn*I (K), and *Xba*I (X).

For the insertion of additional epitopes in loop 5, synthetic complementary oligonucleotides of 39 bp were made (Fig. 2). Annealing of the complementary strands resulted in *Kpn*I-compatible sticky ends. Plasmid pCO20 was digested with *Kpn*I, ligated to a 30-fold molar excess of nonphosphorylated double-stranded oligonucleotide, heated to 65°C and cooled slowly to room temperature to melt out the noncovalently linked strands, redigested with *Kpn*I (insertion of the oligonucleotide removes the *Kpn*I site), and transformed

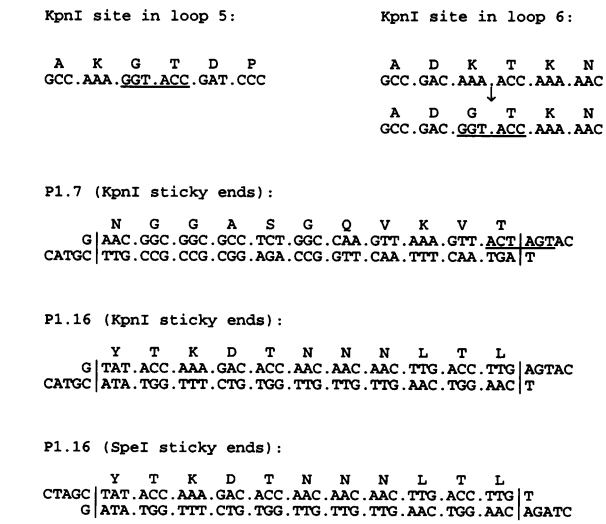

FIG. 2. Oligonucleotides used for the insertion of additional epitopes. Double-stranded oligonucleotides containing *Kpn*I-compatible sticky ends and the sequences of the previously identified P1.7 and P1.16 epitopes (18) were synthesized and ligated into the *Kpn*I site in loop 5 and into the *Kpn*I site introduced as indicated into loop 6 by PCR mutagenesis. Insertion of either oligonucleotide removes the *Kpn*I site; insertion of the P1.7 oligonucleotide introduces a *Spe*I site (underlined), which was subsequently used for insertion of a P1.16 oligonucleotide with *Spe*I-compatible sticky ends.

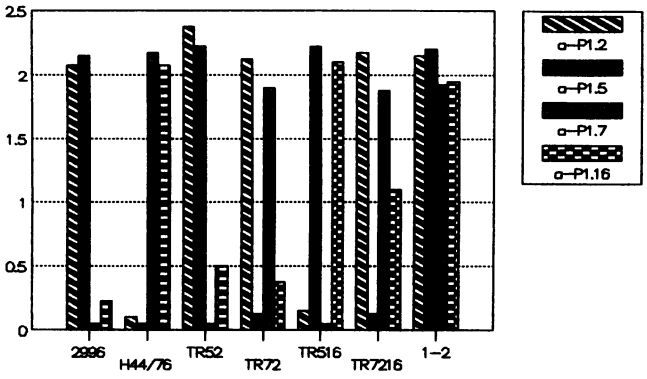

FIG. 3. Binding of MAbs to whole cells of derivatives of H44/76 expressing hybrid class 1 proteins. The extinction at 450 nm is indicated for each strain with four subtype-specific MAbs.

to *E. coli* NM522. To test whether the insertion was present in the correct orientation, transformants were grown in the presence of IPTG to induce the LacZ-PorA fusion protein. The resulting cell lysates were tested by Western blotting (immunoblotting) with MAbs specific for P1.2 and the inserted epitope P1.7 or P1.16. For the insertion of both epitopes, a pCO20 derivative with an inserted P1.7 oligonucleotide was digested with *Spe*I and ligated in the same way to a P1.16 oligonucleotide with *Spe*I sticky ends (Fig. 2). This results in a tandem insertion of both oligonucleotides. The presence of both P1.7 and P1.16 epitopes (in addition to P1.2) was again verified by Western blotting.

Insertions in loop 6 were made in exactly the same way, starting from plasmid pPH204, which has a *Kpn*I site located in the part of *porA* encoding loop 6 (Fig. 2). This plasmid was derived from pCO20 in the following way. First, the *Kpn*I site in loop 5 was removed by digestion with *Kpn*I, flushing with T4 DNA polymerase, and insertion of a 10-bp nonphosphorylated *Bam*HI linker (5'-CGGGATCCCG-3'). Second, a *Kpn*I site was introduced in loop 6 by PCR mutagenesis. PCR products were generated with primer mp1 (5'-GGA GGTACCAAAAACAGTACGACCGAAATT-3') plus M13 reverse sequencing primer (5'-CAGGAAACAGCTATGAC-3') on pCO2, consisting of the C-terminal part of *porA*, and with mp2 (5'-GGAGGTACCGTCGGCATTTTCAGACAA ATCCAA-3') plus M13 reverse sequencing primer on pCO20 with the *Bam*HI linker insertion, consisting of the N-terminal part. These PCR products were then digested with *Kpn*I (underlined sequence) and used to reassemble the 1.9-kb *Eco*RI fragment. The resulting plasmid, pPH204, differs from pCO20 by the replacement of the *Kpn*I site in loop 5 by a *Bam*HI site and the insertion of a new *Kpn*I site in loop 6.

Transformation of meningococci. Allelic replacement of the meningococcal *porA* gene with constructs made in *E. coli* was done by transformation with undigested plasmid DNA as described previously (32). Transformants expressing the selected epitope of the incoming *porA* gene were identified by using a colony blot procedure (32) with one of the following MAbs (18, 31): MN16C13F4 (P1.2), MN22A9.19 (P1.5), MN14C11.6 (P1.7), and MN5C11G (P1.16). Expression of class 1 epitopes on whole cells of the constructed strains was quantitated by enzyme-linked immunosorbent assay (ELISA) (1). The plates were read at 450 nm on a model EL312e Bio-Kinetics reader (Bio-Tek Instruments).

Immunological methods. OMCs were isolated from meningococci grown in liquid medium by sarcosyl extraction as described previously (31). The protein composition was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (16). Western immunoblotting of cell lysates and OMCs with subtype-specific MAbs was done as described elsewhere (23). For immunization, outbred 14- to 17-g female NIH mice were injected subcutaneously with 2.5 μg of OMC protein in saline and 0.5 mg of $AlPO_4$ in a volume of 0.5 ml. This procedure was repeated after 4 weeks, and the animals were bled at week 6. Bactericidal activity of the sera was determined by using human complement from an immunoglobulin-deficient patient (22).

RESULTS

Construction of isogenic meningococcal strains expressing hybrid class 1 outer membrane proteins. To investigate further the role of the class 1 variable regions in the induction of bactericidal antibodies by OMCs, allelic replacement by transformation was used to construct a set of isogenic derivatives of strain H44/76 differing only in the *porA* gene. Transformants were found at a frequency of approximately 10^{-3} to 10^{-4} when plasmid DNA was used, which is sufficiently high for direct identification in a colony blot with subtype-specific MAbs. The plasmids used for transformation carry different fragments of *porA* alleles and are shown in Fig. 1. First, transformation with plasmids pCO14 and pCO3 resulted in strains TR52 and TR72 (Table 1), in which the 7,16 allele has been changed to 5,2 and 7,2, respectively. Strain TR52 was subsequently transformed to 5,16 with plasmid pCO6. In this way, we obtained derivatives of H44/76 which expressed each of the four epitope combinations P1.7,16, P1.5,2, P1.7,2, and P1.5,16. Expres-

TABLE 2. Bactericidal titers of mouse antisera against OMCs containing hybrid class 1 proteins

Immunizing OMC	Bactericidal titer against test strain[a]:			
	2996 (2b:P1.5,2)	MC50 (nt:P1.16)	TR52 (15:P1.5,2)	H44/76 (15:P1.7,16)
H44/76 (15:P1.7,16)	<1:2	>1:2,048	1:256	>1:2,048
2996 (2b:P1.5,2)	>1:2,048	<1:2	>1:2,048	1:8
1-2 (−:P1.7,16,5,2)	>1:2,048	1:1,024	>1:2,048	>1:2,048
TR52 (15:P1.5,2)	>1:2,048	<1:2	>1:2,048	<1:2
TR72 (15:P1.7,2)	1:2,048	<1:2	>1:2,048	1:256
TR516 (15:P1.5,16)	1:128	1:512	1:256	1:1,024
TR7216 (15:P1.7,2,16)	1:2,048	<1:2	>1:2,048	1:512
Control	<1:2	<1:2	1:32	<1:2

[a] Mice were immunized with 2.5 μg of OMC protein. For each immunization, eight mice were used and their sera were pooled. Bactericidal titers are shown as the highest serum dilution that killed half of the cells of the indicated test strain.

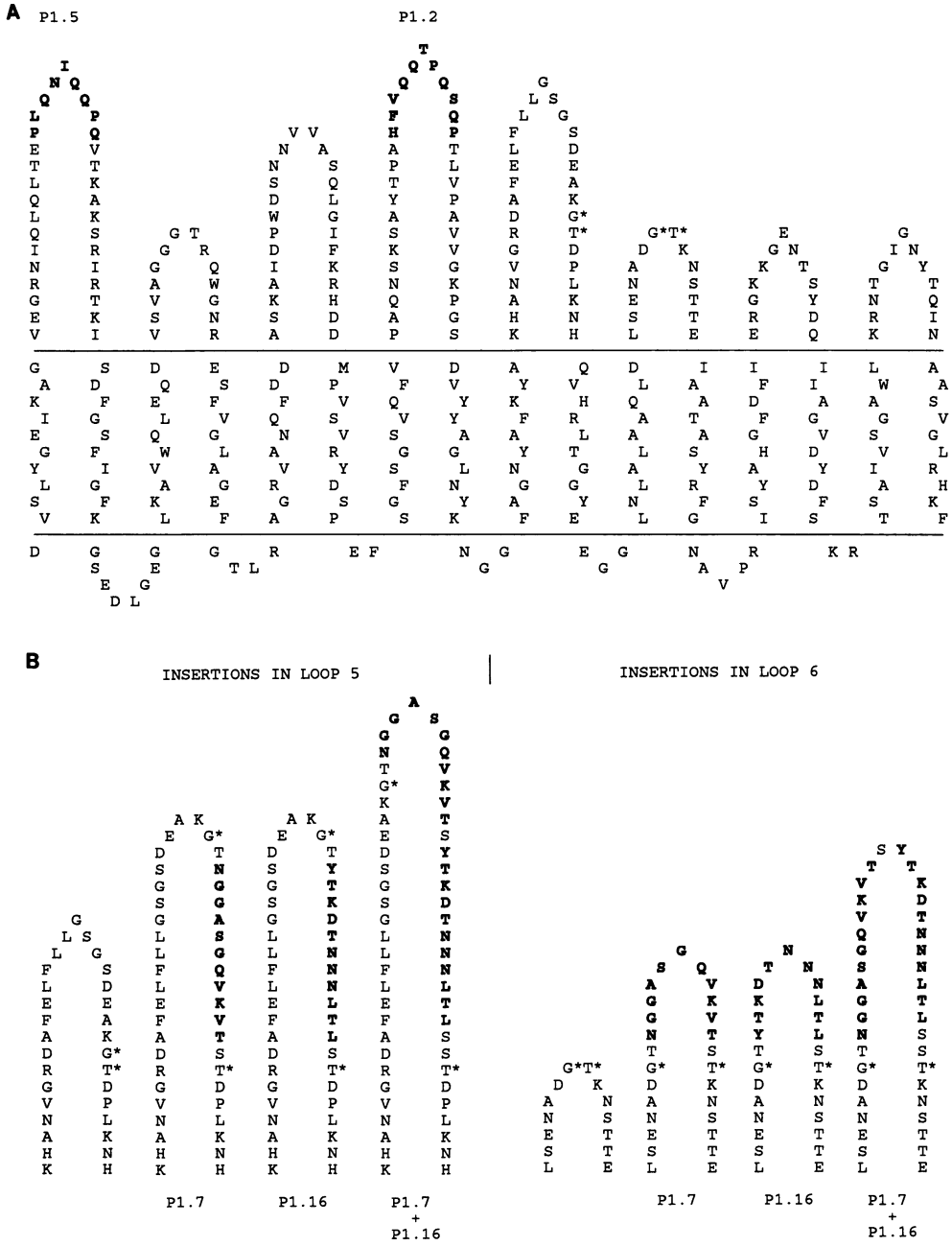

FIG. 4. Insertion of additional epitopes into loops 5 and 6 of class 1 protein. (A) Topology model for strain 2996 (adapted from reference 31). (B) The various insertions into loops 5 and 6 of this protein. The residues marked with asterisks represent points of insertion. Residues shown in boldface represent the P1.5, P1.2, P1.7, and P1.16 epitopes as identified previously (18, 31).

sion of these epitope combinations was verified with a whole-cell ELISA (Fig. 3).

In addition, a strain expressing a P1.7,2,16 epitope combination was obtained by transformation with a derivative of pCO20 in which a 39-bp oligonucleotide has been inserted into the *Kpn*I site, which is located in the part of the *porA* gene encoding loop 5 (Fig. 2). Transformation of TR72 with this plasmid and selection with the P1.16-specific MAb resulted in strain TR7216, expressing the P1.16 epitope as a 13-amino-acid residue insertion into loop 5, in addition to the P1.7 and P1.2 epitopes in loops 1 and 4, respectively. Although the P1.16-specific MAb was able to bind to this protein at the cell surface, the whole-cell ELISA values obtained were considerably lower than those for a normal P1.16 strain (Fig. 3). Isolation of OMCs and analysis by SDS-PAGE demonstrated that all hybrid class 1 proteins thus obtained were present in the outer membrane in normal amounts (not shown). Neither H44/76 nor any other one of this particular set of derivatives expressed any *opa* gene in a significant amount.

Immunization with OMCs containing hybrid class 1 proteins. Mice were immunized with OMC preparations from

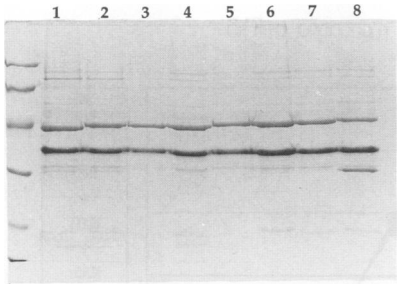

FIG. 5. SDS-PAGE analysis of OMCs from strains with additional class 1 epitopes. Shown are TR52 (lane 1), H44/76 (lane 2), K007 (lane 3), K016s (lane 4), K716 (lane 5), J007 (lane 6), J016 (lane 7), and J716 (lane 8). Molecular mass markers of 94, 67, 43, 30, 20.1, and 14.4 kDa are present in the leftmost lane.

the set of strains described above and from 2996 (the P1.5,2 reference strain) and the bivalent vaccine strain 1-2 (32). The last is a class 3 protein-deficient derivative of H44/76 that expresses two different class 1 proteins, i.e., of subtypes P1.7,16 and P1.5,2 (32); it was included as a positive control for class 1-specific bactericidal activity. The other strains used for this immunization differ only in class 1 protein; the other major OMC component, class 3 protein of serotype 15, was shared among them. The resulting antisera were tested for bactericidal activity against strains H44/76, TR52, 2996, and MC50 (Table 2). High bactericidal activity was found only when the immunizing OMCs and the test strain shared at least one of their class 1 variable regions. The only exception was strain TR52 with the antisera induced by H44/76 OMCs; this strain was also the only one that showed some bactericidal activity with the control sera. In all cases, higher levels of bactericidal antibodies were induced by the second than by the first variable region. This difference is more pronounced for the P1.5/P1.2 than for the P1.7/P1.16 comparison. Strain TR7216 produced bactericidal titers similar to those produced by strain TR72; this also occurred when MC50 was used as the test strain. Apparently, the P1.16 epitope in loop 5 is not capable of inducing bactericidal antibodies against P1.16 in its normal position in loop 4.

Insertion of additional epitopes into surface-exposed loops of class 1 protein. The topology models for neisserial porins predict eight surface-exposed loops (31). In class 1 protein, the protective epitopes are located in loops 1 and 4. In an attempt to construct meningococcal strains carrying more than the normal two subtype determinants, we inserted additional epitopes into loops 5 and 6 in the following way. Plasmid pCO20 carries a 1.9-kb fragment encoding the C-terminal part of the *porA* gene from strain 2996, starting at residue 100 and fused in frame to the *lacZ'* gene (see Materials and Methods for details of plasmid constructions). Oligonucleotides with *Kpn*I-compatible sticky ends were inserted into the unique *Kpn*I site of this plasmid, a site located in the part of the gene encoding loop 5 (Fig. 2). For insertion into loop 6, site-specific mutagenesis was used to construct plasmid pPH204, with the *Kpn*I site located in the part encoding the apex of loop 6 instead of loop 5 (Fig. 2). The constructs were subsequently used for transformation of strain TR52, with selection for one of the newly inserted epitopes in a colony blot. In this way, we obtained a set of strains which carry the P1.5 epitope in loop 1, P1.2 in loop 4, and P1.7 and P1.16 (separately or in combination) in either loop 5 or loop 6 (Table 1 and Fig. 4). The insertions had a length of either 13 or 26 residues. All strains showed a normal amount of class 1 protein in the outer membrane, as demonstrated by SDS-PAGE of OMCs (Fig. 5). In a whole-cell ELISA, the level of binding of P1.5- and P1.2-specific MAbs to the cell surface was the same as with the parent strain TR52 (Fig. 6). Compared with strain H44/76, the P1.7 and P1.16 epitopes gave a variable amount of binding dependent on the particular construct (Fig. 6). Surface exposure of all additional epitopes could be demonstrated, although binding to P1.16 in strain J716 was very weak. When the transformant containing an insertion of P1.16 in loop 5 was streaked for purification, the colony blot also showed a single aberrant colony with more intense MAb binding. This variant was also isolated. In the whole-cell ELISA, an almost twofold difference in binding to P1.16 was found between these two variants, termed K016z and K016s, for weak and strong reactivity, respectively (Fig. 6). On SDS-PAGE, these two variants were identical with respect to the level of class 1 expression and its electrophoretic mobility (not shown).

TABLE 3. Bactericidal titers of mouse antisera against OMCs containing additional epitopes in class 1 protein

Immunizing OMC	Bactericidal titer against test strain[a]:	
	H44/76 (15:P1.7,16)	2996 (2b:P1.5,2)
H44/76 (15:P1.7,16)	>1:2,048	<1:2
TR52 (15:P1.5,2)	1:128	>1:2,048
K007 (15:P1.5,2,7)	1:512	>1:2,048
K016s (15:P1.5,2,16)	1:1,024	1:2,048
K716 (15:P1.5,2,7,16)	1:256	1:2,048
J007 (15:P1.5,2,7)	1:256	>1:2,048
J016 (15:P1.5,2,16)	1:512	>1:2,048
J716 (15:P1.5,2,7,16)	1:2,048	>1:2,048
Control	<1:2	<1:2

[a] Mice were immunized with 2.5 μg of OMC protein. For each immunization, eight mice were used and their sera were pooled. Bactericidal titers are shown as the highest serum dilution that killed half of the cells of the indicated test strain. In a duplicate experiment with the same sera, titers did not differ from those shown by more than a factor of 2.

Immunization with OMCs containing additional epitopes in class 1 protein. Mice were immunized with OMC preparations from the set of strains described above, including the parent strains H44/76 and TR52. These OMCs differ only in class 1 protein, with the exception of J716, which also produces a class 5 protein (Fig. 5). The resulting antisera were tested for bactericidal activity against strains H44/76 and 2996 (Table 3). The insertion of epitopes in loops 5 and 6 of TR52 resulted in a significant increase in bactericidal titer against H44/76 with several constructs, in particular K016s and J716, which almost reached the level of H44/76 itself. Bactericidal titers against strain 2996 remained high in all cases, showing that the immunogenicity of loops 1 and 4 is not impaired. Since the test strains used do not express significant amounts of class 5 protein, its presence in J716 cannot influence the outcome of the bactericidal assay.

DISCUSSION

In a previous study, we have presented a model for the topology of class 1 protein and other neisserial porins (31). It predicted eight surface-exposed loops on a 16-stranded β-sheet transmembrane structure. Similar models have been proposed for PhoE protein and other porins from *E. coli* (29). The recent determination of high-resolution three-dimensional structures for the porin of *Rhodobacter capsulatus*

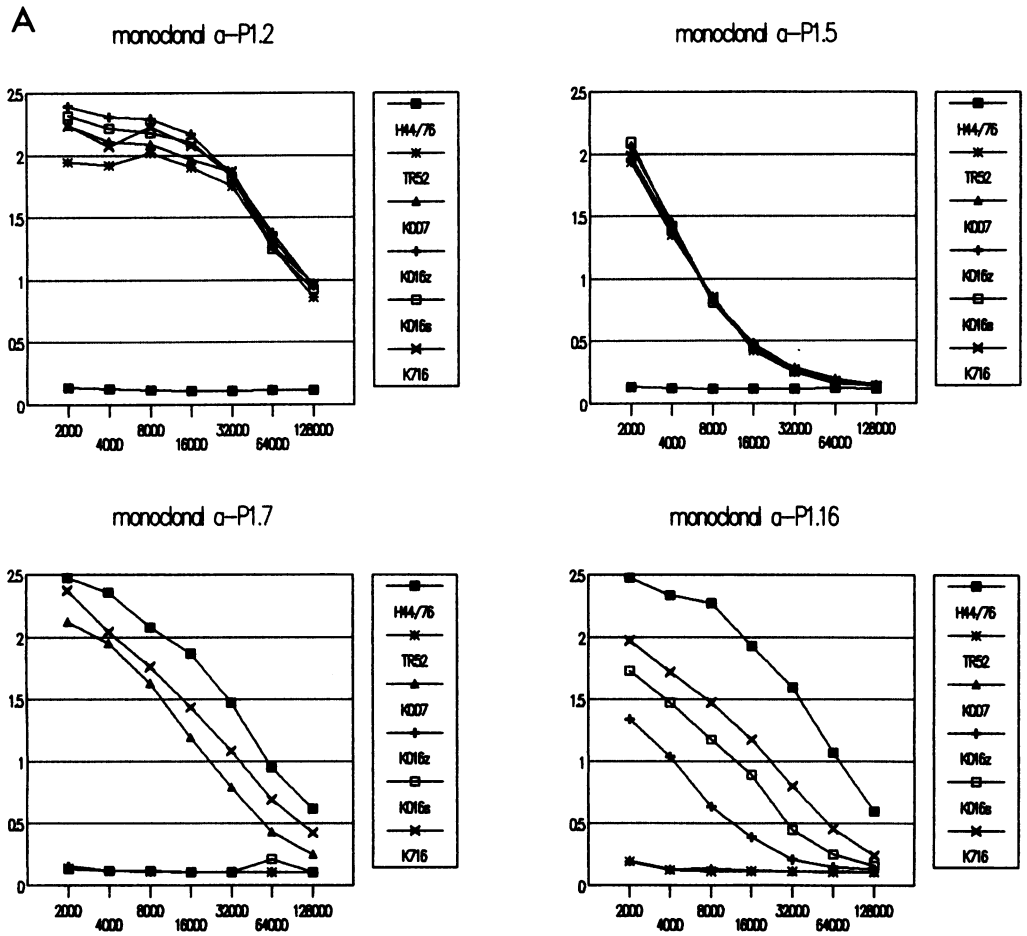

FIG. 6. Binding of MAbs to whole cells of strains carrying additional epitopes in loop 5 (A) or loop 6 (B). For each strain, the extinction at 450 nm is plotted against the MAb dilution used.

(34) and the OmpF and PhoE porins of *E. coli* (8) has confirmed the validity of these models and suggests the existence of a porin fold common to many of these proteins from different bacteria, which show only little sequence homology (14). The present study has confirmed the surface exposure of loops 5 and 6 of class 1 protein, since epitopes inserted here are accessible to MAbs in whole cells. Surface exposure has previously been demonstrated for loops 1 and 4 (31).

After immunization of mice with OMCs, bactericidal activity is much higher against strains with the same class 1 protein than against those with only the same class 3 protein (Table 2) (32). A plausible explanation for this difference is provided by our model, which shows that the length of the surface-exposed loops is much longer in class 1 protein than in other neisserial porins, including the class 3 protein, for which sequence information has only recently become available (6). One can thus envisage that these loops in class 1 protein are more immunogenic and/or more accessible to antibodies once formed.

Sequence diversity among different class 1 subtypes is mainly confined to loops 1 and 4 (31). In addition to completely different versions of these variable regions, sequence heterogeneity of particular subtypes has also been observed (19, 20). Sometimes the same variable regions are found in different combinations in different strains (9, 17, 18). These observations have to be taken into account when the construction of a multivalent vaccine based on the class 1 protein is considered (32). The results presented in this study are important in this context. They show that changing one variable region still allows the correct antibodies to be formed against the other one; in this respect, these two loops thus behave to a large extent independently of each other. In addition, we have demonstrated that bactericidal activity is higher against loop 4 than against loop 1.

We chose loops 5 and 6 of class 1 protein for the insertion of additional epitopes because in the homologous gonococcal porins PIA and PIB, they can bind bactericidal antibodies (31). Also, in class 2 and 3 proteins, these loops show sequence diversity among different serotypes, albeit less pronounced than in the variable regions of class 1 protein (10, 33, 35). The surface exposure of the newly inserted epitopes shows that this is a valid approach for the construction of multivalent class 1 proteins. In a similar way, the PhoE and LamB outer membrane proteins of *E. coli* have been used as carriers of foreign antigenic determinants (2, 15). A different aspect of our work is the need to insert additional epitopes without disturbing the existing ones. This is indeed possible for loops 5 and 6, because (i) these regions are not very important for the induction of bactericidal antibodies, as evidenced by the low or absent bactericidal activity when the immunizing and test strains share loops 5

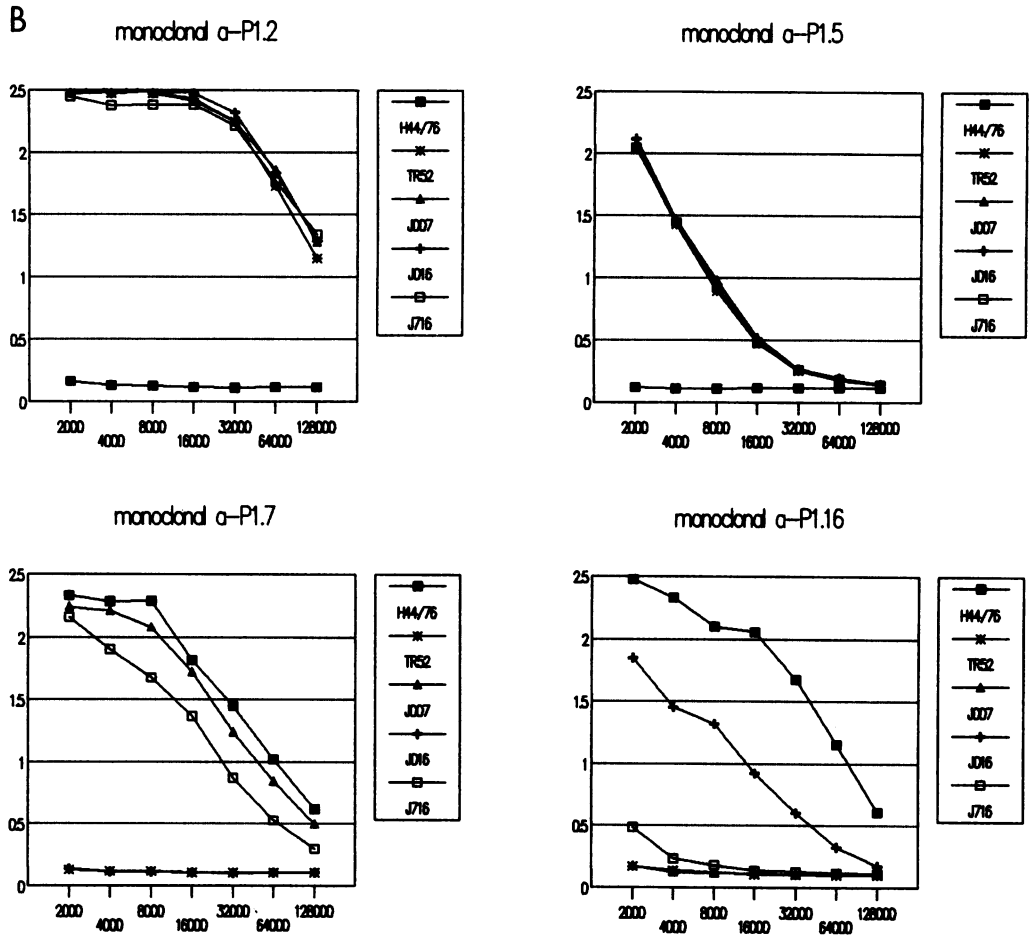

FIG. 6—*Continued.*

and 6 but have different variable regions, (ii) the insertions do not show any effect on the accessibility of loops 1 and 4 to their MAbs, and (iii) the bactericidal response against loops 1 and 4 remains unaffected when insertions are present in loop 5 or 6.

Induction of bactericidal antibodies by additional epitopes is one of the goals of this approach. It was initially expected that this would require the construction of strains showing a level of MAb binding to whole cells similar to the wild-type level. With the P1.7 epitope, this was indeed found for all constructs; with P1.16, binding was more variable. Apparently, the latter is more dependent on a particular configuration. However, induction of bactericidal antibodies is not correlated to MAb binding in the whole-cell ELISA; in strain J716, P1.16 binding is very low and P1.7 binding is not as high as in some other constructs, whereas this strain gave the highest bactericidal titer. Of course, the affinity of a particular MAb for its epitope is probably dependent not only on surface exposure per se but also on the particular conformation of the inserted sequence in a construct; this effect is likely to be different when the induction of antibodies, i.e., immunogenicity instead of antigenicity, is considered. Since the ability of the constructs to induce bactericidal antibodies can apparently not be predicted from the level of MAb binding, empirical construction of the optimal insertion for each individual epitope will be necessary.

Insertion of P1.16 in loop 5 resulted in bactericidal activity against this epitope with strain K016s but not with strain TR7216. In the former strain, a spontaneous variant with a twofold-higher level of P1.16 MAb binding was accidentally obtained and used in the immunization. The nature of this mutation is not known, but it is a likely cause for the observed difference between TR7216 and K016s in bactericidal activity. Since a comparison of K016z and K016s showed no alteration in binding of lipopolysaccharide (LPS)-specific MAbs (not shown) or of P1.5/2-specific MAbs (Fig. 6), a point mutation specifically affecting the P1.16 epitope is the most likely explanation.

As this work has demonstrated, insertion of additional sequences of up to 26 residues in the surface-exposed loops 5 and 6 of class 1 protein while maintaining the immunogenicity of loops 1 and 4 is possible. In addition to inserting meningococcal subtype epitopes, this approach can also be used to insert other useful sequences in class 1 protein without distorting its immunogenicity. For instance, insertion of cysteine residues could allow chemical conjugation of LPS-derived oligosaccharides to a predetermined site at the cell surface-exposed part of the protein.

In the course of this work, transformation of meningococci with cloned *porA* genes was found to be very easy. Transformation frequencies of 10^{-3} to 10^{-4} were routinely obtained, making direct identification in a colony blot possible without the need for use of an antibiotic resistance marker. Transformants thus obtained always resulted from allelic replacement. This high frequency suggests that transformation could also allow strains to change their subtype in

vivo. Indeed, sequence comparison of different *porA* alleles has shown a mosaic composition, reminiscent of exchange of sequence blocks between different strains (9).

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